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SPECIAL ISSUE STEM CELLS

Is stem cell chromosomes stability affected by cryopreservation conditions?

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Introduction

The introduction of the recombinant DNA techniques in the 1970s paved the way to "Gene Therapy" a novel branch of modern medicine. Although gene therapy is still at an experimental stage, it has great potentials since allows to transfer, into organs and tissues, genetic information. However, several technical problems still need to be overcome before this approach takes over conventional pharmacological treatments (Hacein-Bey-Abina et al. 2002; Mullen et al. 1996).

Today stem cells, either embryonic or adult, are acquiring a great deal of attention as they promise, and rightly so, to be new vehicles for gene therapy. Unfortunately, the in vivo genetic instability of stem cells, and even more pronounced for embryonic derived stem cells, limits their widespread use. The prototypic example of adult stem cells, the hematopoietic stem cells have already been used in gene therapy (Aiuti et al. 2002) after being isolated from bone marrow or after their mobilization into peripheral blood. Although adult tissues with high turnover rate are maintained by tissue specific stem cells, they themselves rarely divide. Recently, a related stem cell, the multipotent progenitor cells have been isolated from bone marrow and these can differentiate into multiple lineages (Gregory et al. 2005). Other stem cells have been identified, both in the central nervous system and in the heart, but as of today they have been less characterized and are not easily accessible (Stocum 2005).

A major drawback in using adult stem cells is that it is very difficult to maintain the stem state during ex vivo manipulations. Adult stem cells tend to loose their stem cell properties and become more specialized since they have the tendency to differentiate. Culturing conditions may influence the differentiating capacity of these cells by acting as signal transmitters (Reya et al. 2003; Willert et al. 2003).

On the other hand, embryonic stem cells maintain their capacity to differentiate into derivatives of all three germ layers even after prolonged laboratory growth. They grow rapidly, they are remarkably stable and maintain the in vitro capacity to mature into multiple cell types of the body (Zwaka and Thomson 2005). These characteristics make embryonic stem cells ideal for gene therapy applications. However, while these cells in culturing dishes appear remarkably stable, they may accumulate genetic and epigenetic changes, which may harm the patient.

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While so much is being learned on how to genetically modify stem cells and how to introduce them into a tissue or organ, relatively little is known on how to cryoprotect them from becoming chromosomically unstable, how to maintain them in toxic free agents and in scaffolds ready to be introduced into patients. Indeed sporadic chromosomal abnormalities in human embryonic stem cells have been reported which appear to be more frequent when they are passaged as bulk populations (Draper et al. 2004). It is then important to optimize culturing conditions, cryostorage and monitoring systems to be applied to the newly derived as well as existing cell lines to decipher any genetic and epigenetic alterations, which may have taken place.

Technically, cryobiology is the study of living systems, at any temperature below the standard physiological range. However freezing temperature variations above or below the physiological threshold may be very dangerous if not lethal to all types of cells (Doxey et al. 2006). The therapeutic use of stem and progenitor cells from different organs/systems to treat a variety of human diseases require the development of validated, clinical-grade cellular freeze-thawing procedures to minimize adverse effects or toxicity to patients (Buchanan et al. 2005). To achieve successful results of transplantation and treatment of significant diseases as Parkinson's disease, Alzheimer's disease, leukemia, diabetes, stroke, muscular dystrophy, hepatic and renal failure etc., optimum methods need to be developed on how to obtain stem cells, the way in which they are cultivated, how and for how long they are cryopreserved. Essentially, it would be important to develop protocols for freeze-thawing all clinically relevant cells in a state for immediate use on patients and, most of all, establish ways to minimize adverse responses.

Today, the scientific community is very concerned with the available cryoprotective agents and their effects on the function of the cells. Indeed, a number of non toxic cryopreservation reagents and protocols have been developed by biotech companies dealing with this aspect of clinical research. Particular attention has been given to hematopoietic stem and progenitor cell conditions since they have been used to treat a number of human diseases. However, one aspect of stem cell research, which has not yet been fully explored, is on the chromosomal stability of these cells as a function of duplication time, cryoprotective reagents and storage conditions. The variation in chromosome number is probably the main type of genomic instability recorded and this is usually manifested as loss or gain of whole chromosomes, generally known as aneuploidy (Pathak and Multani 2006). Chromosome instability is a devastating phenomenon underlying several human diseases. Aneuploidy arises from meiotic errors and is almost always found in cancers, but is also associated with aging. Stochastic or spontaneous chromosomal variations in somatic cells appear as low level mosaic aneuploidy which are usually thought to be insignificant and overlooked probably due to unapparent phenotypic effects.

This minireview aims to summarize the present knowledge on the subject.

General aspects of stem cell cryoconservation conditions, cell duplication and chromosomal stability

Currently the standard method for human cell cryopreservation is slow programmed freezing, using medium containing human serum albumin and cryoprotective molecules such as dextran and dimethylsulphoxide (DMSO) at high concentrations. DMSO is an amphipathic molecule and besides causing adverse effects and toxicity to patients (Sauer-Heilborn et al. 2004), is known to cause unexpected changes in cell fate (Edwards et al. 1983; Preisler and Giladi 1975). It is well established that DNA methylation and acethylation control mammalian development and cellular differentiation (Li 2002). DMSO likely affects these epigenetic changes by acting on one or more of Dnmts (DNA methyltransfereases) as well as on enzymes which modify histones (Iwatani et al. 2006). Hypermethylation and hypomethylation may also occur in several diverse genomic and genic loci thus affecting stem cells phenotype.

Keeping cells in industrial freezers at −80 to −130 °C is a routine procedure for cell biologists. However, such storage may induce substantial damage due to maintaining the cells at temperature higher than the glass transition temperature of the sample. Trounson's group demonstrated that current methods of freezing, storage and recovery trigger apoptosis

and spontaneous differentiation of hES with consequent loss of pluripotency (Pera et al. 2000; Reubinoff et al. 2000). Several other groups have also confirmed very low survival of hES cell after slow freezing with DMSO (Heng et al. 2005; Ji et al. 2004; Kim et al. 2004).

A non toxic alternative protectant is trehalose, a glucose disaccharide which has been shown to improve the stability of stem cells during freezing, freeze-drying and air-drying (Rodrigues et al. 2008). Trehalose forms a glass, a crucial process in cell preservation and storage in a dry state even at high temperatures and moisture content. The major problem in using this disaccharide is the impermeability of the plasma membrane leading to considerable difficulty in introducing high concentrations of the polymer in the cell cytoplasm (Buchanan et al. 2004). Major manipulations, like cell permeabilization, need to take place first, but this may affect signalling pathways due to cell receptor and membrane channel damages. Contrary to DMSO, the effects of trehalose on DNA methylation and histone acetylation is still at an embryonic stage.

Other extracellular water soluble cryoprotective macromolecules such as dextran, albumin, modified gelatins, polyvinylpyrrolidone, polyethylene oxide, polyethylene glycol, and hydroxyethyl starch (HES) act at the cell membrane surface increasing the osmolarity of the medium and keeping fewer water molecules in contact with the cell surface. This reduces the formation of ice crystals in the interior of the cell during the freezing process stabilizing the cell membrane and proteins. Due to these characteristic, many laboratories are using a combination of DMSO and HES to cryopreserve stem cells showing better cell recovery (Clapisson et al. 2004); the synergic effect exerted by intracellular and extracellular cryoprotectors lowers the melting temperature of the membrane phospholipids by hydrogen bonding to the phospholipids head-groups, thereby preventing leakage during freezing, drying and rehydration.

Recently, a new slow freezing method by ECM treatment has been proven as reliable and effective cryopreservation method for hES. Kim et al demonstrated that ECMs, as collagene IV and laminin, could provide a platform for multiple signaling mechanisms to maintain ES features after thawing (Kim et al. 2004). Laminin and collagen IV receptors ($\alpha 6\beta 1$ and $\alpha 6\beta 4$ integrins) are highly expressed in stem cells and embryonal carcinoma cells and may be important for the maintenance of the undifferentiated state (Xu et al. 2001) and to keep a normal karyotype even at passage 30 after thawing.

Alternative to slow freezing techniques, vitrification of hES cells by the open-pulled-straw techniques has been reported as effective for their cryopreservation, even if this process could increase the level of cell death and spontaneous differentiation after thawing (Reubinoff et al. 2001). Furthermore, the limited number of hES clumps that could be cryopreserved simultaneously and the potential hazard of transmission of infective agents to the cells limit the use of this technique in medicine (Tedder et al. 1995).

Several studies have shown how freezing/thawing techniques may lead to alteration in DNA replication or, possibly, chromatin structure. This suggests that cryopreservation and prefreezing manipulation might cause an alteration either in the DNA structure or in the DNA repairing mechanism. It has also been suggested that freezing could increase the production of free radicals (Rao and David 1984), which are liable to injure DNA (Leibovitz and Siegel 1980). In any case, the exact target of the complete cycle of freezing/thawing remains to be clarified.

It has been suggested that DNA instability takes over not only during meiotic, but also during mitotic events. Un unequal sister chromatid exchange is involved in a mechanism of gene tandem duplication that occur in some human diseases as well as during cancer transformation. Sister chromatid exchanges (SCEs) involve breakage of both DNA strands, followed by an exchange of whole DNA duplexes. This occurs during S phase and is efficiently induced by mutagens that form DNA adducts or that interfere with DNA replication. The formation of SCEs has been correlated with recombinational repair and the induction of point mutations, gene amplification and cytotoxicity (Carrano et al. 1978).

The SCE test has been performed to study chromosomal abnormalities in frozen-thawed oocytes showing an increased polyploidy rate (Bouquet et al. 1993) as well as increase in aneuploidy (Kola et al. 1988).

The freezing process gives rise to two major problems: morphological and chromosomal abnormalities that lead to alteration in cell function as proliferation, differentiation and ultimately survival.

Freezing and particularly thawing are responsible for ice and gas bubbles formation both intra- and extracellularly inducing abnormal segregation of chromosomes by disruption of spindle microtubules. Nevertheless although cooling the cells slowly avoids intracellular ice buildup, which can cause the rupture of cell membrane, it can result in dehydratation of cells by the formation of extracellular ice. However, the combined use of DMSO and cooling seems to reduce much of these disruptions and have stabilizing effects on microtubules (Vincent et al. 1990).

Recent studies have hypothesized that mitotic-spindle checkpoint, which helps maintain chromosomal integrity during all cell divisions, functions in human and mouse ESCs, but does not initiate apoptosis as it does in somatic cells (Mantel et al. 2007). This allows an unusual tolerance to polyploidy resulting from failed mitosis, which is common in rapidly proliferating cell populations and which is reduced to near-diploid aneuploidy, which is also common in human neoplastic diseases. Thus, the spindle checkpoint is "uncoupled" from apoptosis in ESCs and is a likely source of karyotypic abnormalities. h/mESCs, which do have the molecular machinery for apoptosis, have a remarkable tolerance for mitotic failure-induced polyploidy, a condition rarely observed in most mammalian somatic cells. Freezing procedures and chemical agents could therefore trigger DNA breakage and replication abnormalities that stem cells would tolerate. ESCs, removed from the context of early embryonic development in the preimplantation blastocyst and grown in culture, could remain uncoupled and subject to increased genome damage. Considering that recovery of viable hESCs from freezing is usually very low and their growth rate is very slow, extended culture periods are needed, exerting selective pressure on stem cell populations and leading to expansion of different sub-populations in different laboratories.

If this is so, it would be extremely important to develop new strategies to help maintain genomic fidelity in ESC cultures.

Discussion

In the future, it may be possible to use stem cells derived from different sources (peripheral, foetal, amniotic, embryonic and cancer niches) in transplantation as a mean to treat severe diseases. A major concern regarding the use of these cells has been the possible risk of chromosomal changes occurring during long-term culture and cryoprotectant agents. Indeed, several reports have appeared in the literature which deal with aspects of chromosomal instability of embryonic stem cells (human and mouse) while being cultured continuously in vitro (Inzunza et al. 2004). There are hardly any paper describing the chromosomal stability of peripheral, amniotic fluid, and foetal derived stem or progenitor cells. There is even less literature which deal with the aspect of chromosomal stability as a function of cryoconservation reagents and storage conditions. Certainly telomeres, guanine-rich tandem DNA repeats of the chromosomal end, provide chromosomal stability by protecting the individual chromosome from disintegration, end-to-end fusion and maintain the genomic integrity during somatic cell divisions (Lingner and Cech 1998). Cell replication induces telomeres loss due to diminished capacity of the telomerase (a reverse transcriptase which elongates the telomeric repeats) activity. In somatic cells, in fact, the telomere length gets shortened gradually at each cell division which triggers senescence; in embryonic stem cells, the activity of the telomerase is high thus maintaining their immortality (Hiyama and Hiyama 2007). Several reports have appeared in the literature since the first study on cancer stem cells deriving from haematopoietic malignancies (Bonnet and Dick 1997) correlating chromosomal instability and malignant transformation. Cancer stem cells isolated from haematopoietic malignancies as well as from solid tumours show numeric chromosome imbalance and that telomere attrition is the earliest genetic alteration responsible for the induction of aneuplody (Pathak and Multani 2006).

This minireview highlights the lack of information regarding that effects of different culturing techniques and long-term culture on stem cells chromosomal stability. Since these cells are promising therapeutic tools, then regular and careful analysis of the chromosomes constituting the cell lines is auspicable. Traditional karyotyping, CGH, FISH and PCR can be used for continuous analysis of these cells during cultivation, storage and freezing–thawing. Since the ideal situation is to directly inject stem cells into patients (from the freezer to the

patient) then it is recommended to freeze them at low passage number and in larger batches in order to secure material in case mutations occur in the cell line at a later stage of culture.

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